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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	5.1183
First Named Inventor or Application Identifier	
Satoshi Koizumi, et al.	
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

1. [ ] Fee Transmittal Form *(Submit an original, and a duplicate for fee processing)*
2. [X] Specification — *Total Pages* 38
3. [X] Drawing(s) *(35 USC 113)* — *Total Sheets* 3
4. [X] Oath or Declaration — *Total Pages* 2
   - a. [X] Newly executed (original or copy)
   - b. [ ] Unexecuted for information purposes
   - c. [ ] Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional with Box 17 completed)* ***[Note Box 5 below]***
     - i. [ ] DELETION OF INVENTOR(S) Signed Statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
5. [ ] Incorporation By Reference *(useable if Box 4c is checked)* The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4c, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. [ ] Microfiche Computer Program *(Appendix)*
7. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*
   - a. [X] Computer Readable Copy
   - b. [X] Paper Copy (identical to computer copy)
   - c. [X] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. [X] Assignment Papers (cover sheet & document(s))
9. [ ] 37 CFR 3.73(b) Statement *(when there is an assignee)* [ ] Power of Attorney
10. [ ] English Translation Document *(if applicable)*
11. [X] Information Disclosure Statement (IDS)/PTO-1449 [X] Copies of IDS Citations
12. [X] Preliminary Amendment
13. [X] Return Receipt Postcard (MPEP 503) *(Should be specifically itemized)*
14. [ ] Small Entity Statement(s) [ ] Statement filed in prior application Status still proper and desired
15. [X] Certified Copy of Priority Document(s) *(if foreign priority is claimed)*
16. [X] Other: Claim to Priority.

17. If a CONTINUING APPLICATION, *check appropriate box and supply the requisite information:*
    [ ] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application No. ___/_____

18. CORRESPONDENCE ADDRESS

[X] Customer Number or Bar Code Label — 05514 (Insert Customer No. or Attach bar code label here) — or [ ] Correspondence address below

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Country		Telephone		Fax	

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CLAIMS	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
	TOTAL CLAIMS (37 CFR 1 16(c))	28 -20 =	8	X $ 18.00 =	$144.00
	INDEPENDENT CLAIMS (37 cfr 1.16(b))	1 -3 =	0	X $ 78.00 =	$0.00
	MULTIPLE DEPENDENT CLAIMS (if applicable) (37 CFR 1.16(d))			$260.00 =	$260.00
				BASIC FEE (37 CFR 1.16(a))	$690.00
			Total of above Calculations =		$1094.00
	Reduction by 50% for filing by small entity (Note 37 CFR 1 9, 1 27, 1.28)				
				TOTAL =	$1094.00

19 Small entity status

a. ☐ A Small entity statement is enclosed

b ☐ A small entity statement was filed in the prior nonprovisional application and such status is still proper and desired.

c. ☐ Is no longer claimed.

20. [X] A check in the amount of $ 1094.00 to cover the filing fee is enclosed

21 [X] A check in the amount of $ 40.00 to cover the recordal fee is enclosed.

22. The Commissioner is hereby authorized to credit overpayments or charge the following fees to Deposit Account No 06-1205.

a. [X] Fees required under 37 CFR 1.16.

b. [X] Fees required under 37 CFR 1.17.

c. ☐ Fees required under 37 CFR 1.18.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED	
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SIGNATURE	
DATE	August 25, 2000

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CORRESPONDENCE INFORMATION

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APPLICATION INFORMATION

Title Line One:: PROCESS FOR PRODUCING N-ACETYLNEURAMINIC ACID

Total Drawing Sheets:: 3
Formal Drawings?:: Yes
Application Type:: Utility

Docket Number:: 5.1183
Secrecy Order in Parent Appl.?:: No

REPRESENTATIVE INFORMATION

Representative Customer Number:: 5514

PRIOR FOREIGN APPLICATIONS

Foreign Application One:: 242670/99
Filing Date:: August 30, 1999
Country:: Japan
Priority Claimed:: Yes

5.1183 PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	:	Examiner: Not Yet Assigned
SATOSHI KOIZUMI, ET AL.	)	
	:	Group Art Unit: N/Y/A
Application No.: N/Y/A	)	
	:	
Filed: Currently herewith	)	
	:	
For: PROCESS FOR PRODUCING N-ACETYLNEURAMINIC ACID	) :	August 25, 2000

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to action on the merits, please amend the above-identified application as follows:

IN THE CLAIMS:

Please amend Claims 6-11 as follows.

Claim 6, line 1, change "any of claims 1-5" to --claim 5--.

Claim 7, line 1, change "any of claims 1-6" to --claim 5--.

Claim 8, line 1, change "any of claims 1-7" to --claim 5--.

Claim 9, line 1, change "any of claims 1-8" to --claim 5--.

Claim 10, line 1, change "any of claims 6-9" to --claim 6--.

Claim 11, line 1, delete "8 or 9,".

Please add the following new claims 12-16.

--12. The process according to claim 7, wherein said microorganism belonging to the genus Escherichia is Escherichia coli.

13. The process according to claim 8, wherein said microorganism belonging to the genus Escherichia is Escherichia coli.

14. The process according to claim 9, wherein said microorganism belonging to the genus Escherichia is Escherichia coli.

15. The process according to claim 8, wherein said microorganism belonging to the genus Corynebacterium is Corynebacterium ammoniagenes, Corynebacterium glutamicum or Corynebacterium acetoacidophilum.

16. The process according to claim 9, wherein said microorganism belonging to the genus Corynebacterium is Corynebacterium ammoniagenes, Corynebacterium glutamicum or Corynebacterium acetoacidophilum.--

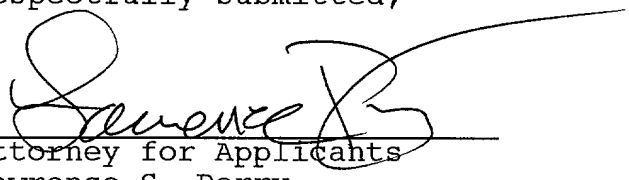
REMARKS

Claims 6-11 have been amended to correct their dependency and conformity with accepted U.S. practice. No new matter has been added.

Entry hereof is earnestly solicited.

Applicants' undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should continue to be directed to our below listed address.

Respectfully submitted,



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Title: PROCESS FOR PRODUCING N-ACETYLNEURAMINIC ACID

PROCESS FOR PRODUCING N-ACETYLNEURAMINIC ACID

Background of the Invention

The present invention relates to a process for producing N-acetylneuraminic acid by using a microorganism having N-acetylneuraminic acid aldolase activity or N-acetylneuraminic acid synthetase activity.

It is known that N-acetylneuraminic acid can be produced by extraction, decomposition or by use of enzymes.

An example of a known method by extraction is extraction from a nest of sea swallows, etc. [Carbohydrate Research, 56, 423 (1977)].

An example of a known method by decomposition is decomposition of colominic acid, which is an N-acetylneuraminic acid polymer produced by Escherichia coli, etc. [J. Biochem., 82, 1425 (1977)].

Known methods utilizing enzymes include the following: methods using N-acetylneuraminic acid aldolase, pyruvic acid and N-acetylmannosamine [J. Am. Chem. Soc., 110, 6481 (1988); J. Am. Chem. Soc., 110, 7159 (1988)]; a method using N-acetylneuraminic acid aldolase, pyruvic acid and N-acetylglucosamine under alkaline conditions (U.S. Patent No. 5,665,574); methods using N-acetylneuraminic acid aldolase, N-acetylglucosamine 2-epimerase, pyruvic acid and N-acetylglucosamine [Angew. Chem. Int. Ed. Eng., 30, 827 (1991); Carbohydrate Research, 306, 575 (1998)]; and methods using N-acetylneuraminic acid synthetase, phosphoenolpyruvic acid and N-acetylmannosamine [Japanese Published Unexamined Patent Application No. 4961/98; Glycobiology, 7, 697 (1997)].

The above methods for producing N-acetylneuraminic acid require complicated operations or expensive materials such as pyruvic acid and phosphoenolpyruvic acid, and an economical method for producing N-acetylneuraminic acid has not been established yet.

So far, there has been no report describing or suggesting that N-acetylneuraminic acid can be produced by utilizing a

culture of a microorganism or a treated matter thereof.

As for the N-acetylneuraminic acid aldolase, those derived from animals and plants are known, and it is known that microorganisms belonging to the genus Escherichia have the activity of this enzyme. Also known is the presence of the gene encoding this enzyme, nanA, in an Escherichia coli strain [Nucleic Acids Res., 13, 8843 (1985)].

N-Acetylneuraminic acid synthetase is known to be present in microorganisms belonging to the genera Escherichia, Neisseria and Streptococcus, etc., and it is known that an Escherichia coli strain has the gene encoding this enzyme, neuB [J. Bacteriol., 177, 312 (1995)].

N-Acetylglucosamine 2-epimerase is known to be present in pigs and rats. The properties of the enzyme derived from pig have been investigated [Biochemistry, 17, 3363 (1970)] and the gene encoding the enzyme [J. Biol. Chem., 271, 16294 (1996)] has been obtained. So far, no microorganism having the activity of this enzyme is known.

As to the production of pyruvic acid, a process for producing pyruvic acid by using a mutant of Escherichia coli is known [Biosci. Biotech. Biochem., 58, 2164 (1994)].

As to the production of phosphoenolpyruvic acid, a process for producing phosphoenolpyruvic acid by using microorganisms of Saccharomyces, etc. is known (Japanese Published Unexamined Patent Application No. 197778/94).

An object of the present invention is to provide a process for economically producing N-acetylneuraminic acid without using expensive materials such as pyruvic acid and phosphoenolpyruvic acid. A further object of the present invention is to provide a process for producing N-acetylneuraminic acid without using expensive N-acetylmannosamine.

Summary of the Invention

The present inventors have made an intensive investigation to attain the above objects and have found that

N-acetylneuraminic acid can be efficiently produced from inexpensive materials by utilizing a microorganism which is capable of producing pyruvic acid or phosphoenolpyruvic acid. The present invention has been completed based on this finding.

The present invention relates to the following (1)-(11).

(1) A process for producing N-acetylneuraminic acid which comprises:

allowing (i) a culture of a microorganism having N-acetylneuraminic acid aldolase activity or N-acetylneuraminic acid synthetase activity, or a treated matter of the culture, (ii) a culture of a microorganism capable of producing pyruvic acid or a treated matter of the culture when a microorganism having N-acetylneuraminic acid aldolase activity is used in (i) above, or a culture of a microorganism capable of producing phosphoenolpyruvic acid or a treated matter of the culture when a microorganism having N-acetylneuraminic acid synthetase activity is used in (i) above, (iii) N-acetylmannosamine, and (iv) an energy source which is necessary for the formation of pyruvic acid or phosphoenolpyruvic acid to be present in an aqueous medium to form and accumulate N-acetylneuraminic acid in the aqueous medium; and

recovering N-acetylneuraminic acid from the aqueous medium.

(2) The process according to the above (1) wherein said N-acetylmannosamine is produced by allowing a culture of a microorganism having N-acetylglucosamine 2-epimerase activity or a treated matter of the culture and N-acetylglucosamine to be present in an aqueous medium to form and accumulate N-acetylmannosamine in the aqueous medium.

(3) The process according to the above (2) wherein said microorganism having N-acetylglucosamine 2-epimerase activity carries a recombinant DNA composed of a DNA fragment comprising DNA encoding N-acetylglucosamine

2-epimerase and a vector.

(4) The process according to the above (3) wherein said DNA encoding N-acetylglucosamine 2-epimerase is DNA derived from a microorganism belonging to the genus Synechocystis.

(5) The process according to the above (3) or (4) wherein said DNA encoding N-acetylglucosamine 2-epimerase is selected from the group consisting of:

(a) DNA encoding a protein having the amino acid sequence shown in SEQ ID NO: 1; and

(b) DNA having the nucleotide sequence shown in SEQ ID NO: 2.

(6) The process according to any of the above (1)-(5) wherein said microorganism having N-acetylneuraminic acid aldolase activity is a microorganism belonging to the genus Escherichia or Corynebacterium.

(7) The process according to any of the above (1)-(6) wherein said microorganism having N-acetylneuraminic acid synthetase activity is a microorganism belonging to a genus selected from the group consisting of Escherichia, Neisseria and Streptococcus.

(8) The process according to any of the above (1)-(7) wherein said microorganism capable of producing pyruvic acid is a microorganism belonging to a genus selected from the group consisting of Escherichia, Corynebacterium and Saccharomyces.

(9) The process according to any of the above (1)-(8) wherein said microorganism capable of producing phosphoenolpyruvic acid is a microorganism belonging to a genus selected from the group consisting of Escherichia, Corynebacterium and Saccharomyces.

(10) The process according to any of the above (6)-(9) wherein said microorganism belonging to the genus Escherichia is Escherichia coli.

(11) The process according to the above (6), (8) or (9) wherein said microorganism belonging to the genus Corynebacterium is Corynebacterium ammoniagenes, Corynebacterium

glutamicum or Corynebacterium acetoacidophilum.

Brief Description of the Drawings

Fig. 1 shows the steps for constructing plasmid pTA3 expressing N-acetylneuraminic acid aldolase.

Fig. 2 shows the steps for constructing plasmid pYP18 expressing N-acetylneuraminic acid synthetase.

Fig. 3 shows the steps for constructing plasmid pYP16 expressing N-acetylglucosamine 2-epimerase.

[Explanation of Symbols]

Amp^r: Ampicillin resistance gene

P_L: P_L promoter

cI857: cI857 repressor

P_{lac}: lac promoter

slr1975: N-Acetylglucosamine 2-epimerase gene

nanA: N-Acetylneuraminic acid aldolase gene

neuB: N-Acetylneuraminic acid synthetase gene

Detailed Description of the Invention

In the process of the present invention, any microorganism having N-acetylneuraminic acid aldolase activity can be used. For example, microorganisms belonging to the genus Escherichia or Corynebacterium may be used.

Examples of the microorganisms belonging to the genus Escherichia are those of the species Escherichia coli. Examples of the microorganisms belonging to the genus Corynebacterium are those of the species Corynebacterium ammoniagenes, Corynebacterium glutamicum and Corynebacterium acetoacidophilum.

Also useful are transformants with N-acetylneuraminic acid aldolase activity enhanced by recombinant DNA techniques. Examples of such transformants include microorganisms carrying a recombinant DNA comprising nanA gene derived from Escherichia coli [Nucleic Acids Res., 13, 8843 (1985)], specifically, Escherichia coli NM522/pTA3.

Any microorganism having N-acetylneuraminic acid

synthetase activity can be used in the process of the present invention. For example, microorganisms belonging to the genus Escherichia, Neisseria or Streptococcus may be used.

Examples of the microorganisms belonging to the genus Escherichia are those of the species Escherichia coli.

Also useful are transformants with N-acetylneuraminic acid synthetase activity enhanced by recombinant DNA techniques. Examples of such transformants include microorganisms carrying a recombinant DNA comprising neuB gene derived from Escherichia coli [J. Bacteriol., 177, 312 (1995)], specifically, Escherichia coli NM522/pYP18.

Any microorganism capable of producing pyruvic acid can be used in the process of the present invention. Examples of suitable microorganisms are those of the species Escherichia coli, Corynebacterium ammoniagenes, Corynebacterium glutamicum, Corynebacterium acetoacidophilum and Saccharomyces cerevisiae. Also useful are microorganisms with pyruvic acid productivity enhanced by mutagenesis or recombinant DNA techniques. For example, the Escherichia coli mutant described in Biosci. Biotech. Biochem., 58, 2164 (1994) can be used.

Any microorganism capable of producing phosphoenolpyruvic acid can be used in the process of the present invention. Examples of suitable microorganisms are those of the species Escherichia coli, Corynebacterium ammoniagenes, Corynebacterium glutamicum, Corynebacterium acetoacidophilum and Saccharomyces cerevisiae. An example of the microorganism of the species Saccharomyces cerevisiae is the strain described in Japanese Published Unexamined Patent Application No. 197778/94. Also useful are microorganisms with phosphoenolpyruvic acid productivity enhanced by mutagenesis or recombinant DNA techniques.

Any microorganism having N-acetylglucosamine 2-epimerase activity can be used in the process of the present invention. Suitable microorganisms include transformants with N-acetylglucosamine 2-epimerase activity enhanced by

recombinant DNA techniques. Specific examples of such transformants are Escherichia coli carrying the recombinant DNA pEPI1 comprising the N-acetylglucosamine 2-epimerase gene derived from pig (FERM BP-4602: U.S. Patent No. 5,795,767) and Escherichia coli NM522/pYP16 carrying a recombinant DNA comprising the N-acetylglucosamine 2-epimerase gene derived from a microorganism belonging to the genus Synechocystis.

An example of the N-acetylglucosamine 2-epimerase gene derived from a microorganism belonging to the genus Synechocystis is the gene encoding a polypeptide having the amino acid sequence shown in SEQ ID NO: 1 which exists on the chromosome of Synechocystis sp. PCC6803, more specifically, the gene having the nucleotide sequence shown in SEQ ID NO: 2 (slr1975). The polypeptide having the amino acid sequence shown in SEQ ID NO: 1 and the DNA having the nucleotide sequence shown in SEQ ID NO: 2 have been obtained for the first time by the present inventors according to the procedure described later in an example.

A microorganism having N-acetylneuraminic acid aldolase activity and the ability to produce pyruvic acid can be used alone for the production of N-acetylneuraminic acid from N-acetylmannosamine. In cases where the microorganism employed is weak or lacking in any of the above properties, it may be used in combination with a microorganism which can complement such property for producing N-acetylneuraminic acid.

N-Acetylmannosamine useful in the production of N-acetylneuraminic acid includes N-acetylmannosamine preparations (e.g., commercial products) and N-acetylmannosamine prepared from N-acetylglucosamine by chemical reaction under alkaline conditions or by enzymatic conversion using N-acetylglucosamine 2-epimerase. Also useful are preparations containing N-acetylmannosamine formed and accumulated by allowing a culture of a microorganism having N-acetylglucosamine 2-epimerase activity or a treated matter of the culture and N-acetylglucosamine to be present in an

aqueous medium, and N-acetylmannosamine purified from such preparations.

A microorganism having N-acetylneuraminic acid aldolase activity, N-acetylglucosamine 2-epimerase activity and the ability to produce pyruvic acid can be used alone for the production of N-acetylneuraminic acid from N-acetylglucosamine. In cases where the microorganism employed is weak or lacking in any of the above properties, it may be used in combination with a microorganism which can complement such property for producing N-acetylneuraminic acid.

N-Acetylglucosamine useful in the production of N-acetylneuraminic acid includes N-acetylglucosamine preparations (e.g., commercial products).

A microorganism having N-acetylneuraminic acid synthetase activity and the ability to produce phosphoenolpyruvic acid can also be used alone for the production of N-acetylneuraminic acid from N-acetylmannosamine. In cases where the microorganism employed is weak or lacking in any of the above properties, it may be used in combination with a microorganism which can complement such property for producing N-acetylneuraminic acid.

Further, a microorganism having N-acetylneuraminic acid synthetase activity, N-acetylglucosamine 2-epimerase activity and the ability to produce phosphoenolpyruvic acid can be used alone for the production of N-acetylneuraminic acid from N-acetylglucosamine. In cases where the microorganism employed is weak or lacking in any of the above properties, it may be used in combination with a microorganism which can complement such property for producing N-acetylneuraminic acid.

The microorganisms employed in the production of N-acetylneuraminic acid or N-acetylmannosamine may be subjected to reaction to form the product during their growth stage. Alternatively, after the completion of culturing of a microorganism, the resulting culture or a treated matter of the culture may be subjected to reaction.

As described above, microorganisms prepared by using recombinant DNA techniques can be used in the production of N-acetylneuraminic acid or N-acetylmannosamine. Gene manipulating operations such as isolation and purification of plasmid DNA comprising a desired gene from a microorganism carrying the plasmid, cleavage of plasmid DNA with restriction enzymes, isolation and purification of cleaved DNA fragments, enzymatic ligation of DNA fragments, and transformation with recombinant DNA can be carried out according to known methods [e.g., Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989) (hereinafter referred to as Molecular Cloning, Second Edition); Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997) (hereinafter referred to as Current Protocols in Molecular Biology)]. Polymerase chain reaction (hereinafter referred to as PCR) can be carried out by a known method [PCR Protocols, Academic Press (1990)].

A gene concerned in the production of N-acetylneuraminic acid or N-acetylmannosamine can be expressed in a host by preparing a DNA fragment of an appropriate length containing the gene from a DNA fragment containing the gene by use of restriction enzymes or PCR, inserting the prepared DNA fragment into an appropriate expression vector at a site downstream of the promoter, and then introducing the expression vector comprising the above DNA into a host cell suited for the expression vector.

As the host cell, any bacterial cells, yeast cells, etc. which are capable of expressing a desired gene can be used.

The expression vectors that can be employed are those capable of autonomous replication or integration into chromosome in the above host cells and comprising a promoter at a position appropriate for the transcription of a desired DNA.

When a procaryotic cell such as a bacterial cell is used as the host cell, it is preferred that the expression vector for a gene is a recombinant DNA which is capable of autonomous

replication in the procaryotic cell and which comprises a promoter, a ribosome binding sequence, the desired DNA, and a transcription termination sequence. The vector may further comprise a gene regulating the promoter.

Examples of suitable expression vectors are pBTrp2 (Roche Diagnostics), pBTac1 (Roche Diagnostics), pBTac2 (Roche Diagnostics) , pHelix1 (Roche Diagnostics), pKK233-2 (Amersham Pharmacia Biotech), pKK223-3 (Amersham Pharmacia Biotech), pGEX-2T (Amersham Pharmacia Biotech), pSE280 (Invitrogen), pGEMEX-1 (Promega), pQE-8 (QIAGEN), pQE-30 (QIAGEN), pET-3 (Novagen), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 [Agric. Biol. Chem., 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)], pBluescript II SK+ (Stratagene), pBluescript II SK- (Stratagene), pTrS30 [prepared from Escherichia coli JM109/pTrS30 (FERM BP-5407)], pTrS32 [prepared from Escherichia coli JM109/pTrS32 (FERM BP-5408)], pUC19 [Gene, 33, 103 (1985)], pSTV28 (Takara Shuzo Co., Ltd.), pUC118 (Takara Shuzo Co., Ltd.), pPAC31 (WO 98/12343) and pPA1 (Japanese Published Unexamined Patent Application No. 233798/88).

As the promoter, any promoters capable of functioning in host cells such as Escherichia coli can be used. For example, promoters derived from Escherichia coli or phage, such as trp promoter (Ptrp), lac promoter (Plac), P_L promoter, P_R promoter and P_{SE} promoter, SPO1 promoter, SPO2 promoter and penP promoter can be used. Artificially modified promoters such as a promoter in which two Ptrp are combined in tandem, tac promoter, lacT7 promoter and letI promoter, etc. can also be used.

It is preferred to use a plasmid in which the distance between the Shine-Dalgarno sequence (ribosome binding sequence) and the initiation codon is adjusted to an appropriate length (e.g., 6-18 bases).

In the recombinant DNA of the present invention, the transcription termination sequence is not essential for the

expression of the desired DNA, but it is preferred that the transcription termination sequence lie immediately downstream of the structural gene.

Examples of suitable procaryotes are microorganisms belonging to the genera Escherichia, Serratia, Bacillus, Brevibacterium, Corynebacterium, Microbacterium and Pseudomonas, specifically, Escherichia coli XL1-Blue, Escherichia coli XL2-Blue, Escherichia coli DH1, Escherichia coli MC1000, Escherichia coli W1485, Escherichia coli NM522, Escherichia coli JM109, Escherichia coli HB101, Escherichia coli No. 49, Escherichia coli W3110, Escherichia coli NY49, Serratia ficaria, Serratia fonticola, Serratia liquefaciens, Serratia marcescens, Bacillus subtilis, Bacillus amyloliquefaciens, Brevibacterium immariophilum ATCC 14068, Brevibacterium saccharolyticum ATCC 14066, Corynebacterium ammoniagenes, Corynebacterium glutamicum ATCC 13032, Corynebacterium glutamicum ATCC 14067, Corynebacterium glutamicum ATCC 13869, Corynebacterium acetoacidophilum ATCC 13870, Microbacterium ammoniaphilum ATCC 15354 and Pseudomonas sp. D-0110.

Introduction of the recombinant DNA can be carried out by any of the methods for introducing DNA into the above host cells, for example, the method using calcium ion [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], the protoplast method (Japanese Published Unexamined Patent Application No. 248394/88) and electroporation [Nucleic Acids Research, 16, 6127 (1988)].

When a yeast cell is used as the host cell, YEp13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419), pHS19, pHS15, etc. can be used as the expression vector.

As the promoter, any promoters capable of functioning in yeast cells can be used. Suitable promoters include PHO5 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock polypeptide promoter, MF α 1 promoter, CUP 1 promoter, etc.

Examples of suitable host cells are cells of yeast strains

belonging to the genera Saccharomyces, Schizosaccharomyces, Kluyveromyces, Trichosporon, Schwanniomyces, Pichia and Candida, specifically, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Trichosporon pullulans, Schwanniomyces alluvius, Pichia pastoris and Candida utilis.

Introduction of the recombinant DNA can be carried out by any of the methods for introducing DNA into yeast cells, for example, electroporation [Methods in Enzymol., 194, 182 (1990)], the spheroplast method [Proc. Natl. Acad. Sci. USA, 81, 4889 (1984)] and the lithium acetate method [J. Bacteriol., 153, 163 (1983)].

Culturing of the transformant of the present invention can be carried out by conventional methods for culturing the host cell of the transformant.

For the culturing of the transformant prepared by using a procaryotic cell such as Escherichia coli cell or a eucaryotic cell such as a yeast cell as the host cell, any of natural media and synthetic media can be used insofar as it is a medium suitable for efficient culturing of the transformant which contains carbon sources, nitrogen sources, inorganic substances, etc. which can be assimilated by the host used.

As the carbon sources, any carbon sources which can be assimilated by the host can be used. Examples of suitable carbon sources include carbohydrates such as glucose, fructose, sucrose, molasses containing them, starch and starch hydrolyzate; organic acids such as acetic acid and propionic acid; and alcohols such as ethanol and propanol.

As the nitrogen sources, ammonia, ammonium salts of inorganic or organic acids such as ammonium chloride, ammonium sulfate, ammonium acetate and ammonium phosphate, and other nitrogen-containing compounds can be used as well as peptone, meat extract, yeast extract, corn steep liquor, casein hydrolyzate, soybean cake, soybean cake hydrolyzate, and various fermented cells and digested products thereof.

Examples of the inorganic substances include potassium

dihydrogenphosphate, dipotassium hydrogenphosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate and calcium carbonate.

Culturing is usually carried out under aerobic conditions, for example, by shaking culture or submerged spinner culture under aeration, at 15-40° C for 5 hours to 7 days. The pH is maintained at 3.0-9.0 during the culturing. The pH adjustment is carried out by using an inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia, etc.

If necessary, antibiotics such as ampicillin and tetracycline may be added to the medium during the culturing.

When a microorganism transformed with an expression vector comprising an inducible promoter is cultured, an inducer may be added to the medium, if necessary. For example, in the case of a microorganism transformed with an expression vector comprising lac promoter, isopropyl-β-D-thiogalactopyranoside or the like may be added to the medium; and in the case of a microorganism transformed with an expression vector comprising trp promoter, indoleacrylic acid or the like may be added.

The treated matters of a culture include concentrated culture, dried culture, cells obtained by centrifuging the culture, products obtained by treating the cells by various means such as drying, freeze-drying, treatment with a surfactant, ultrasonication, mechanical friction, treatment with a solvent, enzymatic treatment, protein fractionation and immobilization, an enzyme preparation obtained by extracting the cells, etc.

The amount of each microorganism used in the production of N-acetylneuraminic acid or N-acetylmannosamine is 1-500 g/l, preferably 1-300 g/l, as wet cells.

As the energy source which is necessary for the formation of pyruvic acid or phosphoenolpyruvic acid, any substance can be used that promotes the formation of pyruvic acid or phosphoenolpyruvic acid. Examples of preferred substances

are glucose and fructose. The substances may be used usually at a concentration of 10-300 g/l.

Aqueous media useful in the production of N-acetylneuraminic acid or N-acetylmannosamine include water, buffers such as phosphate buffer, carbonate buffer, acetate buffer, borate buffer, citrate buffer and Tris buffer, alcohols such as methanol and ethanol, esters such as ethyl acetate, ketones such as acetone, amides such as acetamide, etc. Also useful is a medium or culture broth of a microorganism used in the process for producing N-acetylneuraminic acid or N-acetylmannosamine.

If necessary, a chelating agent (e.g., phytic acid), a surfactant or an organic solvent may be added in the process for producing N-acetylneuraminic acid or N-acetylmannosamine.

Any surfactant that promotes the formation of N-acetylneuraminic acid or N-acetylmannosamine can be used. Suitable surfactants include nonionic surfactants such as polyoxyethylene octadecylamine (e.g., Nymeen S-215, NOF Corporation), cationic surfactants such as cetyltrimethylammonium bromide and alkyldimethyl benzylammonium chloride (e.g., Cation F2-40E, NOF Corporation), anionic surfactants such as lauroyl sarcosinate, and tertiary amines such as alkyldimethylamine (e.g., Tertiary Amine FB, NOF Corporation), which may be used alone or in combination. The surfactant is usually used at a concentration of 0.1-50 g/l.

As the organic solvent, xylene, toluene, aliphatic alcohols, acetone, ethyl acetate, etc. may be used usually at a concentration of 0.1-50 ml/l.

The reaction for forming N-acetylneuraminic acid or N-acetylmannosamine is carried out in an aqueous medium at pH 5-10, preferably pH 6-8, at 20-50°C for 1-96 hours.

Adenine, adenosine 5'-monotriphosphate (AMP), adenosine 5'-triphosphate (ATP), magnesium sulfate, magnesium chloride, etc. may be added for promoting the reaction. Adenine, AMP and ATP are usually used at a concentration of 0.01-100 mmol/l.

N-Acetylneuraminic acid or N-acetylmannosamine formed in the aqueous medium can be determined by using a carbohydrate analysis system (Dionex) or the like [Anal. Biochem., 189, 151 (1990)].

N-Acetylneuraminic acid or N-acetylmannosamine can be recovered from the reaction mixture by ordinary methods using active carbon, ion-exchange resins, etc.

Certain embodiments of the present invention are illustrated in the following examples. These examples are not to be construed as limiting the scope of the invention.

Example 1

Construction of a Strain Expressing N-Acetylneuraminic Acid Aldolase Gene Derived from *Escherichia coli*

Escherichia coli W3110 (ATCC 27325) was cultured by the method described in Current Protocols in Molecular Biology and the chromosomal DNA of the microorganism was isolated and purified.

The DNA primer shown in SEQ ID NO: 3 and the DNA primer shown in SEQ ID NO: 4 were synthesized by using a DNA synthesizer (Model 8905, PerSeptive Biosystems).

PCR was carried out using the above synthetic DNAs as primers and the chromosomal DNA of *Escherichia coli* W3110 (ATCC 27325) as a template. That is, PCR was carried out by 30 cycles, one cycle consisting of reaction at 94° C for one minute, reaction at 42° C for 2 minutes and reaction at 72° C for 3 minutes, using 40 μl of a reaction mixture comprising 0.1 μg of the chromosomal DNA, 0.5 μmol/l each of the primers, 2.5 units of Pfu DNA polymerase (Stratagene), 4 μl of buffer for Pfu DNA polymerase (10 x) (Stratagene) and 200 μmol/l each of deoxyNTPs.

One-tenth of the resulting reaction mixture was subjected to agarose gel electrophoresis to confirm that the desired fragment was amplified. Then, the remaining reaction mixture was mixed with an equal amount of phenol/chloroform (1vol/1vol) saturated with TE [10 mmol/l Tris-HCl (pH 8.0),

1 mmol/l EDTA], followed by centrifugation. The obtained upper layer was mixed with a two-fold volume of cold ethanol and allowed to stand at -80° C for 30 minutes. The resulting mixture was centrifuged to obtain a DNA precipitate.

The DNA precipitate was dissolved in 20 μl of TE and 5 μl of the solution was subjected to reaction to cleave the DNA with restriction enzymes HindIII and BamHI. DNA fragments were separated by agarose gel electrophoresis and a 1.2 kb fragment was recovered using Gene Clean II Kit (Bio 101). pUC19 DNA [Gene, 33, 103 (1985)] (0.2 μg) was cleaved with restriction enzymes HindIII and BamHI. DNA fragments were separated by agarose gel electrophoresis and a 2.7 kb fragment was recovered in the same manner.

The 1.2 kb fragment and 2.7 kb fragment obtained above were subjected to ligation reaction using a ligation kit at 16° C for 16 hours. Escherichia coli NM522 capable of producing pyruvic acid was transformed using the ligation mixture according to the known method described above, spread on LB agar medium containing 50 μg/ml ampicillin, and cultured overnight at 30° C.

Escherichia coli NM522/pTA3, which is a transformant carrying the N-acetylneuraminic acid aldolase gene, nanA, was obtained from a colony of the transformant that grew on the above medium. A plasmid was extracted from this transformant by a known method to obtain pTA3, which is a plasmid for expression of N-acetylneuraminic acid aldolase gene. The structure of this plasmid was confirmed by digestion with restriction enzymes (Fig. 1).

Example 2

Production of N-Acetylneuraminic Acid

Escherichia coli NM522/pTA3 obtained in Example 1 was inoculated into 125 ml of LB medium containing 50 μg/ml ampicillin in a 1-l Erlenmeyer flask with baffles, followed by culturing at 28° C with stirring (220 r.p.m.) for 17 hours. The resulting culture (125 ml) was inoculated into 2.5 l of

a liquid medium (pH unadjusted) comprising 10 g/l glucose, 12 g/l Bacto-tryptone (Difco Laboratories Inc.), 24 g/l yeast extract (Difco Laboratories Inc.), 2.3 g/l KH_2PO_4, 12.5 g/l K_2HPO_4 and 50 μ g/ml ampicillin in a 5-l jar fermentor. Culturing was carried out at 37° C for 6 hours under the conditions of stirring at 600 r.p.m. and aeration at 2.5 l/min. During the culturing, the pH of culture was maintained at 7.0 with 28% aqueous ammonia. Glucose was added, according to need, in the course of culturing. The resulting culture was centrifuged to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

A reaction mixture (30 ml) comprising 50 g/l Escherichia coli NM522/pTA3 wet cells, 65 g/l fructose, 40 g/l N-acetylmannosamine, 25 g/l KH_2PO_4, 5 g/l $MgSO_4 \cdot 7H_2O$, 5 g/l phytic acid, 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200-ml beaker and subjected to reaction at 32° C for 25 hours with stirring (900 r.p.m.) using a magnetic stirrer. During the reaction, the pH of reaction mixture was maintained at 7.2 with 4 N NaOH, and according to need, fructose and KH_2PO_4 were added to the reaction mixture.

After the completion of reaction, the reaction product was analyzed by using a carbohydrate analysis system (DX-500, Dionex) and it was found that 0.34 g/l N-acetylneuraminic acid was formed and accumulated in the reaction mixture.

Example 3

Construction of a Strain Expressing N-Acetylneuraminic acid synthetase Gene Derived from Escherichia coli

Escherichia coli K235 (ATCC 13027) was cultured by the method described in Current Protocols in Molecular Biology and the chromosomal DNA of the microorganism was isolated and purified.

The DNA primer shown in SEQ ID NO: 5 and the DNA primer shown in SEQ ID NO: 6 were synthesized by using a DNA synthesizer (Model 8905, PerSeptive Biosystems).

PCR was carried out using the above synthetic DNAs as

primers and the chromosomal DNA of Escherichia coli K235 (ATCC 13027) as a template. That is, PCR was carried out by 30 cycles, one cycle consisting of reaction at 94° C for one minute, reaction at 42° C for 2 minutes and reaction at 72° C for 3 minutes, using 40 μl of a reaction mixture comprising 0.1 μg of the chromosomal DNA, 0.5 μmol/l each of the primers, 2.5 units of Pfu DNA polymerase (Stratagene), 4 μl of buffer for Pfu DNA polymerase (10 x) (Stratagene) and 200 μmol/l each of deoxyNTPs.

One-tenth of the resulting reaction mixture was subjected to agarose gel electrophoresis to confirm that the desired fragment was amplified. Then, the remaining reaction mixture was mixed with an equal amount of phenol/chloroform (1vol/1vol) saturated with TE, followed by centrifugation. The obtained upper layer was mixed with a two-fold volume of cold ethanol and allowed to stand at -80° C for 30 minutes. The resulting mixture was centrifuged to obtain a DNA precipitate.

The DNA precipitate was dissolved in 20 μl of TE and 5 μl of the solution was subjected to reaction to cleave the DNA with restriction enzymes ClaI and BamHI. DNA fragments were separated by agarose gel electrophoresis and a 1.1 kb fragment was recovered using Gene Clean II Kit (Bio 101). pPAC31 DNA (WO98/12343) (0.2 μg) was cleaved with restriction enzymes ClaI and BamHI. DNA fragments were separated by agarose gel electrophoresis and a 5.5 kb fragment was recovered in the same manner.

The 1.1 kb fragment and 5.5 kb fragment obtained above were subjected to ligation reaction using a ligation kit at 16° C for 16 hours. Escherichia coli NM522 capable of producing phosphoenolpyruvic acid was transformed using the ligation mixture according to the known method described above, spread on LB agar medium containing 50 μg/ml ampicillin, and cultured overnight at 30° C.

Escherichia coli NM522/pYP18, which is a transformant carrying the N-acetylneuraminic acid synthetase gene, neuB, was obtained from a colony of the transformant that grew on

the above medium. A plasmid was extracted from this transformant by a known method to obtain pYP18, which is a plasmid for expression of N-acetylneuraminic acid synthetase gene. The structure of this plasmid was confirmed by digestion with restriction enzymes (Fig. 2).

Example 4

Production of N-Acetylneuraminic Acid

Escherichia coli NM522/pYP18 obtained in Example 3 was inoculated into 125 ml of LB medium containing 50 μ g/ml ampicillin in a 1-l Erlenmeyer flask with baffles, followed by culturing at 28° C with stirring (220 r.p.m.) for 17 hours. The resulting culture (125 ml) was inoculated into 2.5 l of a liquid medium (pH unadjusted) comprising 10 g/l glucose, 12 g/l Bacto-tryptone (Difco Laboratories Inc.), 24 g/l yeast extract (Difco Laboratories Inc.), 2.3 g/l KH_2PO_4, 12.5 g/l K_2HPO_4 and 50 μ g/ml ampicillin in a 5-l jar fermentor. Culturing was carried out at 37° C for 4 hours and then at 40° C for 3 hours, under the conditions of stirring at 600 r.p.m. and aeration at 2.5 l/min. During the culturing, the pH of culture was maintained at 7.0 with 28% aqueous ammonia. Glucose was added, according to need, in the course of culturing.

The resulting culture was centrifuged to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

A reaction mixture (30 ml) comprising 50 g/l Escherichia coli NM522/pYP18 wet cells, 65 g/l fructose, 40 g/l N-acetylmannosamine, 25 g/l KH_2PO_4, 5 g/l $MgSO_4 \cdot 7H_2O$, 5 g/l phytic acid, 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200-ml beaker and subjected to reaction at 32° C for 19 hours with stirring (900 r.p.m.) using a magnetic stirrer. During the reaction, the pH of reaction mixture was maintained at 7.2 with 4 N NaOH, and according to need, fructose and KH_2PO_4 were added to the reaction mixture.

After the completion of reaction, the reaction product

was analyzed by using a carbohydrate analysis system (DX-500, Dionex) and it was found that 1.4 g/l N-acetylneuraminic acid was formed and accumulated in the reaction mixture.

Example 5

Production of N-Acetylneuraminic Acid

Escherichia coli NM522/pYP18 obtained in Example 3 was cultured according to the method described in Example 2 and the resulting culture was centrifuged to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

Corynebacterium ammoniagenes ATCC 21170 was inoculated into 25 ml of a liquid medium comprising 50 g/l glucose, 10 g/l polypeptone (Nihon Pharmaceutical Industrial Co., Ltd.), 10 g/l yeast extract (Oriental Yeast Co., Ltd.), 5 g/l urea, 5 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 3 g/l K_2HPO_4, 1 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l $CaCl_2 \cdot 2H_2O$, 10 mg/l $FeSO_4 \cdot 7H_2O$, 10 mg/l $ZnSO_4 \cdot 7H_2O$, 20 mg/l $MnSO_4 \cdot 4\text{-}6H_2O$, 20 mg/l L-cysteine, 10 mg/l calcium D-pantothenate, 5 mg/l vitamin B_1, 5 mg/l nicotinic acid and 30 μ g/l biotin (adjusted to pH 7.2 with 10 N NaOH) in a 300-ml Erlenmeyer flask with baffles, followed by culturing at 28° C with stirring (220 r.p.m.) for 24 hours.

The resulting culture (20 ml) was inoculated into 250 ml of a liquid medium having the same composition as above in a 2-l Erlenmeyer flask with baffles, followed by culturing at 28° C with stirring (220 r.p.m.) for 24 hours. The obtained culture was used as a seed culture.

The seed culture (250 ml) was inoculated into 2.25 l of a liquid medium comprising 150 g/l glucose, 5 g/l meat extract (Kyokuto Pharmaceutical Ind. Co., Ltd.), 10 g/l KH_2PO_4, 10 g/l K_2HPO_4, 10 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l $CaCl_2 \cdot 2H_2O$, 20 mg/l $FeSO_4 \cdot 7H_2O$, 10 mg/l $ZnSO_4 \cdot 7H_2O$, 20 mg/l $MnSO_4 \cdot 4\text{-}6H_2O$ (separately sterilized), 15 mg/l β-alanine (separately sterilized), 20 mg/l L-cysteine, 100 μ g/l biotin, 2 g/l urea and 5 mg/l vitamin B_1 (separately sterilized) (adjusted to pH 7.2 with 10 N NaOH) in a 5-l jar fermentor. Culturing was carried out at 32° C for

24 hours under the conditions of stirring at 600 r.p.m. and aeration at 2.5 l/min. During the culturing, the pH of culture was maintained at 6.8 with 28% aqueous ammonia.

The resulting culture was centrifuged to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

A reaction mixture (30 ml) comprising 50 g/l Escherichia coli NM522/pYP18 wet cells, 150 g/l Corynebacterium ammoniagenes ATCC 21170 wet cells, 65 g/l fructose, 40 g/l N-acetylmannosamine, 25 g/l KH_2PO_4, 5 g/l $MgSO_4 \cdot 7H_2O$, 5 g/l phytic acid, 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200-ml beaker and subjected to reaction at 32° C for 6 hours with stirring (900 r.p.m.) using a magnetic stirrer. During the reaction, the pH of reaction mixture was maintained at 7.2 with 4 N NaOH, and according to need, fructose and KH_2PO_4 were added to the reaction mixture.

After the completion of reaction, the reaction product was analyzed by using a carbohydrate analysis system (DX-500, Dionex) and it was found that 3.1 g/l N-acetylneuraminic acid was formed and accumulated in the reaction mixture.

Example 6

Construction of a Strain Expressing N-Acetylglucosamine 2-Epimerase Gene Derived from Synechocystis

Blast Search of Genbank and a similarity search on CyanoBase (http://www.kazusa.or.jp/cyano/), which is a database of the genomic sequence of Synechocystis sp. (PCC6803), were conducted with the amino acid sequence of N-acetylglucosamine 2-epimerase derived from pig [J. Biol. Chem., 271, 16294 (1996)] as a query. As a result, the above amino acid sequence showed a high homology to the sequence derived from Synechocystis sp. (PCC6803) described as a renin-binding protein (slr1975).

Synechocystis sp. (PCC6803) was cultured by the method described in J. Gen. Microbiol., 111, 1 (1979), and the chromosomal DNA of the microorganism was isolated and purified

by the method described in Current Protocols in Molecular Biology.

PCR was carried out according to the method described in Example 1 using the DNAs shown in SEQ ID NOS: 7 and 8 which had been synthesized by using a DNA synthesizer (Model 8905, PerSeptive Biosystems) as primers and the chromosomal DNA of Synechocystis sp. (PCC6803) as a template.

One-tenth of the resulting reaction mixture was subjected to agarose gel electrophoresis to confirm that the desired fragment was amplified. Then, the remaining reaction mixture was mixed with an equal amount of phenol/chloroform (1vol/1vol) saturated with TE.

The resulting mixture was centrifuged and the obtained upper layer was mixed with a two-fold volume of cold ethanol and allowed to stand at -80° C for 30 minutes. The resulting mixture was centrifuged to obtain a DNA precipitate.

The DNA precipitate was dissolved in 20 μl of TE and 5 μl of the solution was subjected to reaction to cleave the DNA with restriction enzymes ClaI and BamHI. DNA fragments were separated by agarose gel electrophoresis and a 1.2 kb fragment was recovered using Gene Clean II Kit (Bio 101).

pPAC31 DNA (0.2 μg) was cleaved with restriction enzymes ClaI and BamHI. DNA fragments were separated by agarose gel electrophoresis and a 5.5 kb fragment was recovered in the same manner.

The 1.2 kb fragment and 5.5 kb fragment obtained above were subjected to ligation reaction using a ligation kit at 16° C for 16 hours.

Escherichia coli NM522 was transformed using the ligation mixture according to the known method described above, spread on LB agar medium containing 50 μg/ml ampicillin, and cultured overnight at 30° C.

Escherichia coli NM522/pYP16, which is a transformant carrying the DNA encoding N-acetylglucosamine 2-epimerase derived from Synechocystis sp., was obtained from a colony of the transformant that grew on the above medium. A plasmid was

extracted from this transformant by a known method to obtain expression plasmid pYP16. The structure of this plasmid was confirmed by digestion with restriction enzymes (Fig. 3).

Example 7

Production of N-Acetylneuraminic Acid

Escherichia coli NM522/pTA3 obtained in Example 1 was cultured according to the method described in Example 2 and the resulting culture was centrifuged to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

Escherichia coli NM522/pYP16 obtained in Example 6 was inoculated into 125 ml of LB medium containing 50 μg/ml ampicillin in a 1-l Erlenmeyer flask with baffles, followed by culturing at 28° C with stirring (220 r.p.m.) for 17 hours. The resulting culture (125 ml) was inoculated into 2.5 l of a liquid medium (pH unadjusted) comprising 10 g/l glucose, 12 g/l Bacto-tryptone (Difco Laboratories Inc.), 24 g/l yeast extract (Difco Laboratories Inc.), 2.3 g/l KH_2PO_4, 12.5 g/l K_2HPO_4 and 50 μg/ml ampicillin in a 5-l jar fermentor. Culturing was carried out at 30° C for 4 hours and then at 40° C for 3 hours, under the conditions of stirring at 600 r.p.m. and aeration at 2.5 l/min. During the culturing, the pH of culture was maintained at 7.0 with 28% aqueous ammonia. Glucose was added, according to need, in the course of culturing.

The resulting culture was centrifuged to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

Corynebacterium ammoniagenes ATCC 21170 was cultured according to the method described in Example 5 and the resulting culture was centrifuged to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

A reaction mixture (30 ml) comprising 50 g/l Escherichia coli NM522/pTA3 wet cells, 50 g/l Escherichia coli NM522/pYP16

wet cells, 150 g/l Corynebacterium ammoniagenes ATCC 21170 wet cells, 65 g/l fructose, 180 g/l N-acetylglucosamine, 25 g/l KH_2PO_4, 5 g/l $MgSO_4 \cdot 7H_2O$, 5 g/l phytic acid, 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200-ml beaker and subjected to reaction at 32° C for 24 hours with stirring (900 r.p.m.) using a magnetic stirrer. During the reaction, the pH of reaction mixture was maintained at 7.2 with 4 N NaOH, and according to need, fructose and KH_2PO_4 were added to the reaction mixture.

After the completion of reaction, the reaction product was analyzed by using a carbohydrate analysis system (DX-500, Dionex) and it was found that 1.0 g/l N-acetylneuraminic acid was formed and accumulated in the reaction mixture.

Example 8

Production of N-Acetylneuraminic Acid

Escherichia coli NM522/pYP18 obtained in Example 3 and Escherichia coli NM522/pYP16 obtained in Example 6 were cultured according to the methods described in Examples 4 and 7, respectively, and the resulting cultures were centrifuged to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

A reaction mixture (30 ml) comprising 50 g/l Escherichia coli NM522/pYP16 wet cells, 50 g/l Escherichia coli NM522/pYP18 wet cells, 65 g/l fructose, 180 g/l N-acetylglucosamine, 25 g/l KH_2PO_4, 5 g/l $MgSO_4 \cdot 7H_2O$, 5 g/l phytic acid, 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200-ml beaker and subjected to reaction at 32° C for 11 hours with stirring (900 r.p.m.) using a magnetic stirrer. During the reaction, the pH of reaction mixture was maintained at 7.2 with 4 N NaOH, and according to need, fructose and KH_2PO_4 were added to the reaction mixture.

After the completion of reaction, the reaction product was analyzed by using a carbohydrate analysis system (DX-500, Dionex) and it was found that 1.3 g/l N-acetylneuraminic acid was formed and accumulated in the reaction mixture.

Example 9

Production of N-Acetylneuraminic Acid

Escherichia coli NM522/pYP18 obtained in Example 3 and Escherichia coli NM522/pYP16 obtained in Example 6 were cultured according to the methods described in Examples 4 and 7, respectively, and the resulting cultures were centrifuged to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

Corynebacterium ammoniagenes ATCC 21170 was cultured according to the method described in Example 5 and the resulting culture was centrifuged to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

A reaction mixture (30 ml) comprising 50 g/l Escherichia coli NM522/pYP16 wet cells, 50 g/l Escherichia coli NM522/pYP18 wet cells, 150 g/l Corynebacterium ammoniagenes ATCC 21170 wet cells, 65 g/l fructose, 180 g/l N-acetylglucosamine, 25 g/l KH_2PO_4, 5 g/l $MgSO_4 \cdot 7H_2O$, 5 g/l phytic acid, 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200-ml beaker and subjected to reaction at 32° C for 24 hours with stirring (900 r.p.m.) using a magnetic stirrer. During the reaction, the pH of reaction mixture was maintained at 7.2 with 4 N NaOH, and according to need, fructose and KH_2PO_4 were added to the reaction mixture.

After the completion of reaction, the reaction product was analyzed by using a carbohydrate analysis system (DX-500, Dionex) and it was found that 4.3 g/l N-acetylneuraminic acid was formed and accumulated in the reaction mixture.

Example 10

Production of N-Acetylneuraminic Acid

Escherichia coli NM522/pYP18 obtained in Example 3 and Escherichia coli NM522/pYP16 obtained in Example 6 were cultured according to the methods described in Examples 4 and 7, respectively, and the resulting cultures were centrifuged

to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

Corynebacterium ammoniagenes ATCC 21170 was cultured according to the method described in Example 5 and the resulting culture was centrifuged to obtain wet cells. The wet cells could be stored at -20° C and could be used after thawing, according to need.

A reaction mixture (30 ml) comprising 50 g/l Escherichia coli NM522/pYP16 wet cells, 50 g/l Escherichia coli NM522/pYP18 wet cells, 150 g/l Corynebacterium ammoniagenes ATCC 21170 wet cells, 100 g/l glucose, 180 g/l N-acetylglucosamine, 5 g/l adenine, 15 g/l KH_2PO_4, 5 g/l $MgSO_4 \cdot 7H_2O$, 5 g/l phytic acid, 4 g/l Nymeen S-215 and 10 ml/l xylene was put into a 200-ml beaker and subjected to reaction at 32° C for 22 hours with stirring (900 r.p.m.) using a magnetic stirrer. During the reaction, the pH of reaction mixture was maintained at 7.2 with 4 N NaOH, and according to need, glucose and KH_2PO_4 were added to the reaction mixture.

After the completion of reaction, the reaction product was analyzed by using a carbohydrate analysis system (DX-500, Dionex) and it was found that 12.3 g/l N-acetylneuraminic acid was formed and accumulated in the reaction mixture.

SEQUENCE LISTING

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<120> Process for producing N-acetylneuraminic acid

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What is claimed is:

1. A process for producing N-acetylneuraminic acid which comprises:
allowing (i) a culture of a microorganism having N-acetylneuraminic acid aldolase activity or N-acetylneuraminic acid synthetase activity, or a treated matter of the culture, (ii) a culture of a microorganism capable of producing pyruvic acid or a treated matter of the culture when a microorganism having N-acetylneuraminic acid aldolase activity is used in (i) above, or a culture of a microorganism capable of producing phosphoenolpyruvic acid or a treated matter of the culture when a microorganism having N-acetylneuraminic acid synthetase activity is used in (i) above, (iii) N-acetylmannosamine, and (iv) an energy source which is necessary for the formation of pyruvic acid or phosphoenolpyruvic acid to be present in an aqueous medium to form and accumulate N-acetylneuraminic acid in the aqueous medium; and
recovering N-acetylneuraminic acid from the aqueous medium.

2. The process according to claim 1, wherein said N-acetylmannosamine is produced by allowing a culture of a microorganism having N-acetylglucosamine 2-epimerase activity or a treated matter of the culture and N-acetylglucosamine to be present in an aqueous medium to form and accumulate N-acetylmannosamine in the aqueous medium.

3. The process according to claim 2, wherein said microorganism having N-acetylglucosamine 2-epimerase activity carries a recombinant DNA composed of a DNA fragment comprising DNA encoding N-acetylglucosamine 2-epimerase and a vector.

4. The process according to claim 3, wherein said DNA encoding N-acetylglucosamine 2-epimerase is DNA derived from a microorganism belonging to the genus Synechocystis.

5. The process according to claim 3 or 4, wherein said DNA encoding N-acetylglucosamine 2-epimerase is selected from the group consisting of:

(a) DNA encoding a protein having the amino acid sequence shown in SEQ ID NO: 1; and

(b) DNA having the nucleotide sequence shown in SEQ ID NO: 2.

6. The process according to any of claims 1-5, wherein said microorganism having N-acetylneuraminic acid aldolase activity is a microorganism belonging to the genus Escherichia or Corynebacterium.

7. The process according to any of claims 1-6, wherein said microorganism having N-acetylneuraminic acid synthetase activity is a microorganism belonging to a genus selected from the group consisting of Escherichia, Neisseria and Streptococcus.

8. The process according to any of claims 1-7, wherein said microorganism capable of producing pyruvic acid is a microorganism belonging to a genus selected from the group consisting of Escherichia, Corynebacterium and Saccharomyces.

9. The process according to any of claims 1-8, wherein said microorganism capable of producing phosphoenolpyruvic acid is a microorganism belonging to a genus selected from the group consisting of Escherichia, Corynebacterium and Saccharomyces.

10. The process according to any of claims 6-9, wherein said microorganism belonging to the genus Escherichia is Escherichia coli.

11. The process according to claim 6, 8 or 9, wherein said microorganism belonging to the genus Corynebacterium is

Corynebacterium ammoniagenes, Corynebacterium glutamicum or Corynebacterium acetoacidophilum.

ABSTRACT OF THE DISCLOSURE

The present invention provides a process for economically producing N-acetylneuraminic acid without using expensive materials such as pyruvic acid and phosphoenolpyruvic acid. The process comprises: allowing (i) a culture of a microorganism having N-acetylneuraminic acid aldolase activity or N-acetylneuraminic acid synthetase activity, or a treated matter of the culture, (ii) a culture of a microorganism capable of producing pyruvic acid or a treated matter of the culture, or a culture of a microorganism capable of producing phosphoenolpyruvic acid or a treated matter of the culture, (iii) N-acetylmannosamine, and (iv) an energy source which is necessary for the formation of pyruvic acid or phosphoenolpyruvic acid to be present in an aqueous medium to form and accumulate N-acetylneuraminic acid in the aqueous medium; and recovering N-acetylneuraminic acid from the aqueous medium.

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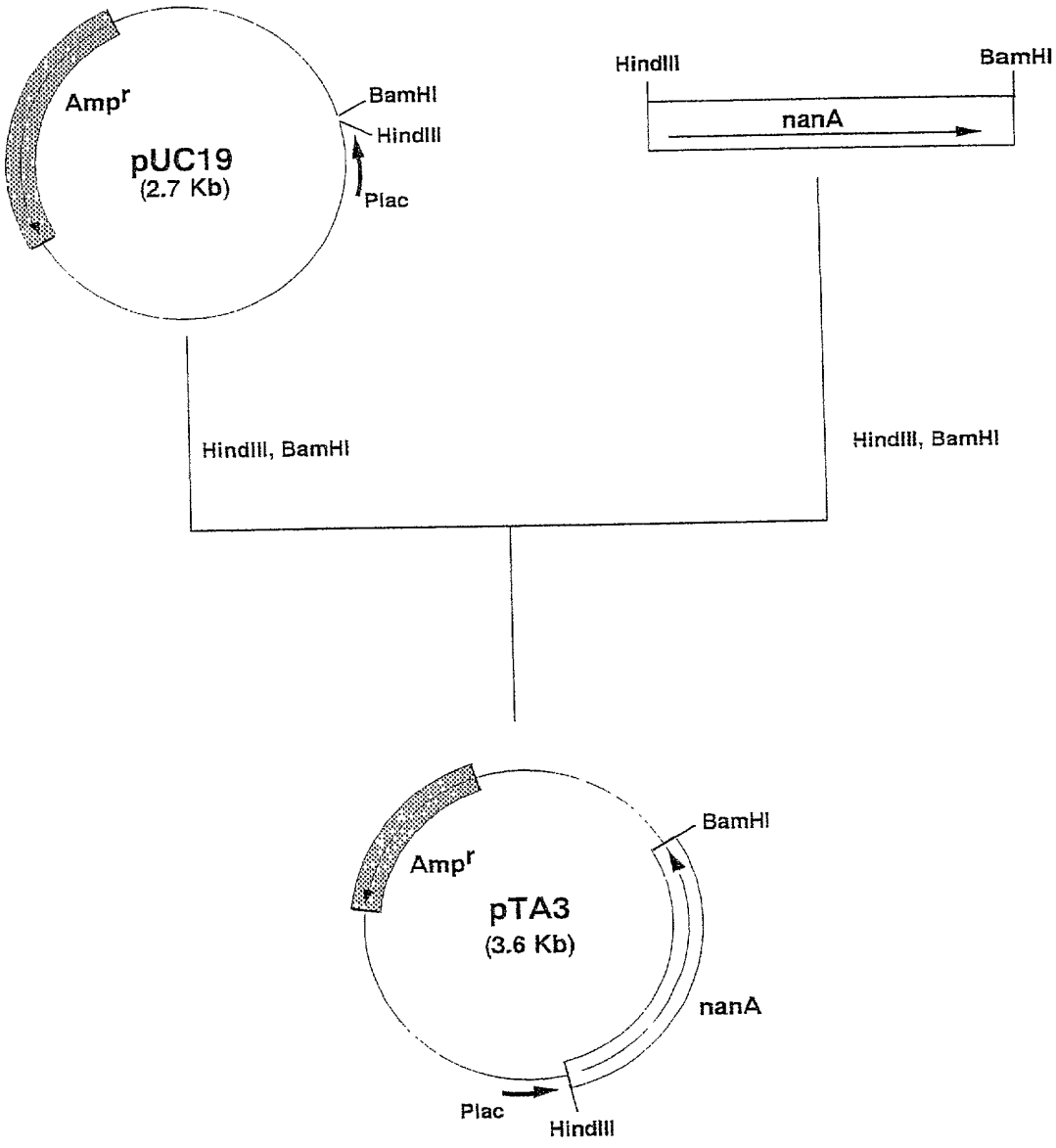
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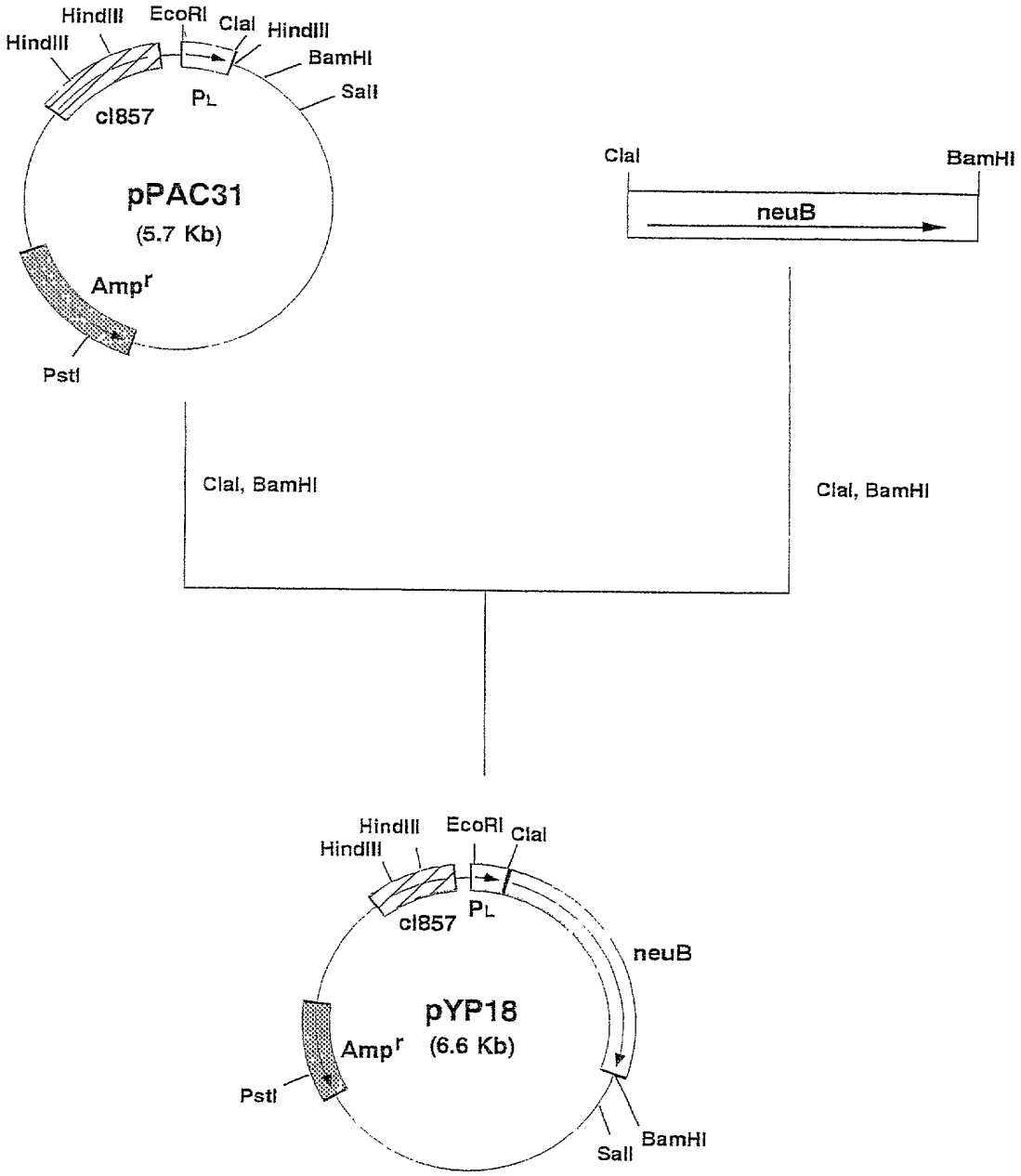
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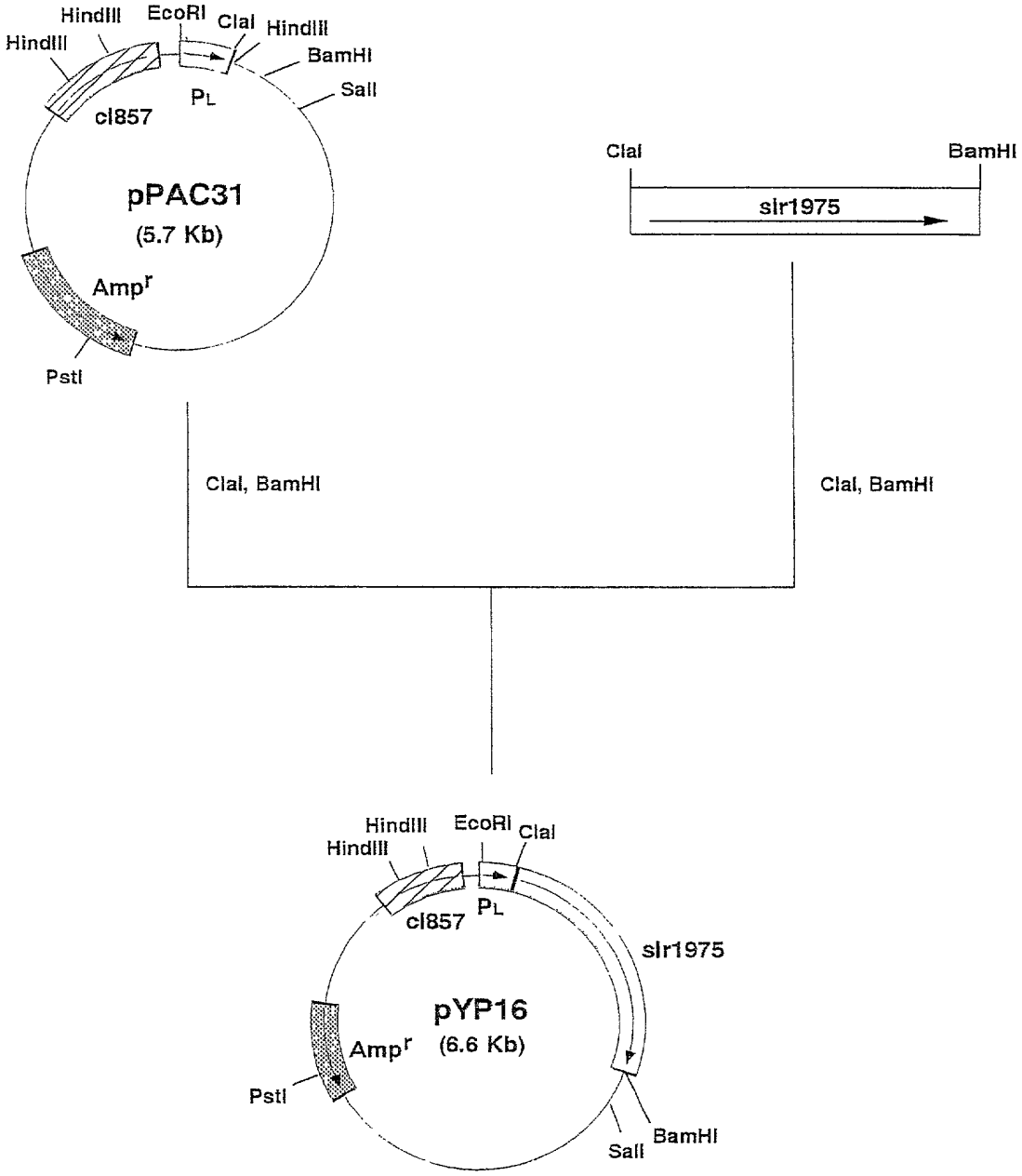
Fig. 3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COMBINED DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
(Page 1)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled PROCESS FOR PRODUCING N-ACETYLNEURAMINIC ACID ______________________________,

the specification of which [X] is attached hereto [ ] was filed on ______________________ as United States Application No. or PCT International Application No. ______________________ and was amended on ______________________ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b), of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designates at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed:

Country	Application No.	Filed (Day/Mo./Yr.)	(Yes/No) Priority Claimed
Japan	242670/99	30 August 1999	Yes

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Application No.	Filed (Day/Mo./Yr.)	Status (Patented, Pending, Abandoned)

I hereby appoint the practitioners associated with the firm and Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to the address associated with that Customer Number:

FITZPATRICK, CELLA, HARPER & SCINTO
Customer Number: 05514

COMBINED DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
(Page 2)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Sole or First Inventor Satoshi Koizumi
Inventor's Signature Satoshi Koizumi
Date August 21, 2000 Citizen/Subject of JAPAN
Residence Tokyo, Japan
Post Office Address c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome, Machida-shi, Tokyo 194-8533 Japan

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Inventor's Signature Kazuhiko Tabata
Date August 18, 2000 Citizen/Subject of JAPAN
Residence Hofu-shi, Japan
Post Office Address c/o Technical Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 1-1, Kyowa-cho, Hofu-shi, Yamaguchi 747-8522 Japan

Full Name of Third Joint Inventor, if any Tetsuo Endo
Inventor's Signature Tetsuo Endo
Date August 21, 2000 Citizen/Subject of JAPAN
Residence Tokyo, Japan
Post Office Address c/o Tokyo Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 6-6, Asahi-machi 3-chome, Machida-shi, Tokyo 194-8533 Japan

Full Name of Fourth Joint Inventor, if any Akio Ozaki
Inventor's Signature Akio Ozaki
Date August 18, 2000 Citizen/Subject of JAPAN
Residence Tokyo, Japan
Post Office Address c/o Technical Research Laboratories, KYOWA HAKKO KOGYO CO., LTD., 1-1, Kyowa-cho, Hofu-shi, Yamaguchi 747-8522 Japan

5.1183 PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	:	Examiner: Not Yet Assigned
SATOSHI KOIZUMI, ET AL.	)	
	:	Group Art Unit: N/Y/A
Application No.: N/Y/A	)	
	:	
Filed: Currently herewith	)	
	:	
For: PROCESS FOR PRODUCING N-ACETYLNEURAMINIC ACID	) :	August 24, 2000

Assistant Commissioner for Patents
Washington, D.C. 20231

SUBMISSION OF COMPUTER READABLE FORM
UNDER 37 C.F.R. § 1.821(e)

Sir:

Applicants submit herewith a computer readable form under 37 C.F.R. § 1.821(e). The content of the computer readable form and the Sequence Listing filed herewith are the same.

Applicants' undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should continue to be directed to our below listed address.

Respectfully submitted,

Attorney for Applicants
Lawrence S. Perry
Registration No. 31,865

FITZPATRICK, CELLA, HARPER & SCINTO
30 Rockefeller Plaza
New York, New York 10112-3801
Facsimile: (212) 218-2200

SEQUENCE LISTING

<110> Satoshi KOIZUMI
Kazuhiko TABATA
Tetsuo ENDO
Akio OZAKI

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1152
Trp Lys Gly Cys Phe His Val Pro Arg Ala Leu Trp Leu Cys Ala Glu
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Thr Leu Gln Leu Pro Val Ser
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36